FORM PTO-1390 (Modified) (REV 10-95) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 19191.0002

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR): TO BE ASSIGNED 09/091578

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US96/20577	19 December 1996	21 December 1995

TITLE OF INVENTION

TARGETED THERAPEUTIC OR DIAGNOSTIC AGENTS AND METHODS OF MAKING AND USING SAME

APPLICANT(S) FOR DO/EO/US

Madison, Edwin L.
Smith, Jeffrey W.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ A copy of the International Search Report (PCT/ISA/210).
8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 18 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
    A **SECOND** or **SUBSEQUENT** preliminary amendment.
16. ☐ A substitute specification.
17. ☐ A change of power of attorney and/or address letter.
18. ☒ Certificate of Mailing by Express Mail
19. ☒ Other items or information:

Sequence listing, sequence listing diskette, check in the amount of $1,702.00, Certificate of Express Mailing No. EL031816978US and return postcard.

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
TO BE ASSIGNED	**PCT/US96/20577**	**19191.0002**

20. The following fees are submitted:.	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :		
☐ Search Report has been prepared by the EPO or JPO **$930.00**		
☒ International preliminary examination fee paid to USPTO (37 CFR 1.482) **$720.00**		
☐ No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) **$790.00**		
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO **$1,070.00**		
☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) **$98.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$720.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$130.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	55 - 20 =	35	x $22.00	**$770.00**	
Independent claims	4 - 3 =	1	x $82.00	**$82.00**	
Multiple Dependent Claims **(check if applicable).**			☐	**$0.00**	
TOTAL OF ABOVE CALCULATIONS =				**$1,702.00**	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) **(check if applicable).** ☐				**$0.00**	
SUBTOTAL =				**$1,702.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**	
TOTAL NATIONAL FEE =				**$1,702.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐				**$0.00**	
TOTAL FEES ENCLOSED =				**$1,702.00**	
				Amount to be: refunded	$
				charged	$

☒ A check in the amount of **$1,702.00** to cover the above fees is enclosed.

☐ Please charge my Deposit Account No. in the amount of to cover the above fees.
A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **14-0629** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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Mary L. Miller
SIGNATURE

Mary L. Miller
NAME

39,303
REGISTRATION NUMBER

19 June 1998
DATE

ATTORNEY DOCKET NO. 19191.0002

APPLICANT OR PATENTEE: EDWIN L. MADISON and JEFFREY W. SMITH
FOR: "TARGETED THERAPEUTIC OR DIAGNOSTIC AGENTS AND METHODS OF MAKING AND USING SAME"

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION: THE SCRIPPS RESEARCH INSTITUTE
ADDRESS OF ORGANIZATION: 10550 North Torrey Pines Road
La Jolla, California 92037

TYPE OF ORGANIZATION:

[ ] University or other institution of higher education
(Name of state)
(Citation of statute)

[X] Tax exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3))

[ ] Non-profit scientific or educational under statute of state of the United States of America
(Name of state)
(Citation of statute)

[ ] Would qualify as tax exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c) (3)) if it were located in The United States of America

[ ] Would qualify as nonprofit scientific or educational under statute of state of The United States of America if it were located in The United States of America
(Name of state)
(Citation of statute)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9 (e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled **"TARGETED THERAPEUTIC OR DIAGNOSTIC AGENTS AND METHODS OF MAKING AND USING SAME"** by inventors EDWIN L. MADISON and JEFFREY W. SMITH described in the specification in the provisional patent application Serial No. 60/009,028, filed December 21, 1995 and in the specification of the U.S. national phase application, filed June 19, 1998, from International Patent Application No. PCT/US96/20577, filed December 19, 1996.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by

any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required for each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME _____
ADDRESS _____

[ ]INDIVIDUAL [ ]SMALL BUSINESS CONCERN [ ]NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: Douglas A. Bingham

TITLE IN ORGANIZATION: Vice President and General Counsel

ADDRESS OF PERSON SIGNING: THE SCRIPPS RESEARCH INSTITUTE
10550 North Torrey Pines Road
La Jolla, California 92037

SIGNATURE: _____ DATE: 8/20/98

Targeted Therapeutic or Diagnostic Agents and Methods of Making and Using Same

This invention was made with government support under Grants RO1 HL52475, PO1 HL31950, R01 CA56483 and R01 AR42750 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to the field of targeting therapeutic or diagnostic entities to a site in a subject. Thus the present invention relates to targeted delivery of therapeutic agents, such as proteins and nucleic acids, among others, and to targeting diagnostic agents to useful sites in a subject

Background Art

Development of the ability to create novel protein-protein interactions promises to provide important new therapeutic agents, novel strategies to target existing therapeutic or diagnostic agents to specific sites, and unique tools and reagents for the study of key biological processes. Such advances in protein engineering may also provide seminal information about how proteins interact. One strategy for manipulating protein/protein interactions is to employ random or nearly random (e.g., alanine scanning) (1) site-directed mutagenesis to identify amino acid residues critical for binding affinity and specificity. Cumbersome, large scale mutagenesis efforts followed by laborious, time consuming assays of individual mutated proteins can sometimes extend this approach to create new molecular interactions. For example, every residue in human growth hormone was scanned for activity prior to re-engineering the molecule to bind the prolactin receptor (2), and a similar strategy facilitated the construction of a variant of IL-3 that binds the monomeric α receptor with higher affinity than it binds the IL-3 $\alpha\beta$ receptor (3). An important current challenge, therefore, is to create more rapid

and efficient strategies to engineer proteins with new binding properties. A recent approach which can obviate the need to perform large numbers of binding assays with individual mutated proteins is biopanning using phage-display systems. Although originally conceived as a means of screening vast numbers of peptide motifs (4), it is now apparent that whole proteins and domains within proteins can be manipulated using the phage selection strategy (5). Phage display itself, however, is also often subject to severe limitations that prevent it from being a completely general approach; many proteins, for example, cannot be displayed on the surface of phage in a biologically active form, and significant problems with proteolysis of proteins displayed on phage are also frequently experienced.

Another strategy that has been used to modify protein/protein interactions is the addition, deletion, or substitution of entire domains within proteins (6-12). Although it is often successful, this strategy provides an extremely low resolution picture of protein/protein interactions; consequently, manipulation of entire domains is often followed by the construction and analysis of numerous point mutants as described above. Severe limitations also arise if a protein domain of interest carries more than one important biological activity; maintaining one activity (e.g., functionally significant domain/domain interactions) while altering another (e.g., high affinity binding to a co-factor or receptor) can be problematic.

The present invention is designed to overcome both of these limitations. In the present method, biologically active structures are identified in one or more protein contexts that are well behaved in phage display studies and then grafted into other proteins of interest. Such a system would eliminate the need to create new libraries for each protein studied. Specifically, the present invention provides a method to transfer between proteins not entire domains but rather defined structural elements within protein domains, or mimetics thereof. The present invention demonstrates grafting flexible protein loop structures (13) which often guide protein binding phenomena. Flexible loops are found on the surface of most protein modules and exist as stretches of 4-20 amino acids that connect regions of defined secondary structure. Although crystallographic and NMR studies show that these loops are usually less well defined

than helices and β-sheets, their conformational freedom is normally restricted substantially compared to free peptides. Consequently, the binding activities of surface loops in proteins usually differ significantly from those of the corresponding linear amino acid sequence (14).

To substantially increase the speed and efficiency by which protein engineering can be used to confer novel binding activities to a selected protein, the present method adopts a strategy that combines phage display and loop grafting. In one embodiment of the present invention, amino acids in a surface loop within the epidermal growth factor (EGF) domain of tissue type plasminogen activator were replaced with residues forming one CDR of a monoclonal antibody that was directed against the adhesive integrin receptor $\alpha_{IIb}\beta_3$ and had been subjected to mutagenesis and "affinity maturation" or "optimization" using a phage display system. The resulting variant of t-PA, LG-t-PA, bound $\alpha_{IIb}\beta_3$ with nanomolar affinity, possessed full activity towards both synthetic and natural substrates, and was stimulated normally by the co-factor fibrin. Because of its novel integrin-binding properties, this new variant of t-PA may display enhanced thrombolytic potency toward the platelet rich thrombi that precipitate acute myocardial infarction.

The resulting variant of t-PA, LG-t-PA, provides an improved thrombolytic agent for the treatment of acute myocardial infarction and other thromboembolic disorders. Efforts to target plasminogen activators to blood clots, by conjugating the enzyme to antibodies or Fab fragments of antibodies directed against either fibrin or platelet integrins, have been previously reported and have often demonstrated that this strategy can enhance the thrombolytic potency of the plasminogen activator both *in vitro* and *in vivo*. For example, chemical conjugation of Mab 7E3 (anti-IIb-IIIa) to urokinase yielded a molecule that was 25-fold more potent in lysing platelet rich clots than wild type urokinase (36). LG-t-PA, therefore, due to its novel binding properties, can be used for enhanced potency towards the platelet rich arterial thrombi which precipitate acute myocardial infarction.

SUMMARY OF THE INVENTION

The present invention provides a targeted therapeutic or diagnostic agent comprising (a) a therapeutic or diagnostic functional entity linked to (b) an isolated peptide mimetic that specifically binds a selected target. The invention additionally provides a targeted therapeutic or diagnostic agent comprising (a) a therapeutic or diagnostic functional entity linked to (b) an isolated, optimized, high-affinity polyamino acid that specifically binds a selected target. The invention further provides a targeted therapeutic or diagnostic agent comprising (a) a therapeutic or diagnostic functional entity linked to (b) an isolated protein surface loop that specifically binds a selected target, wherein the protein surface loop is not endogenous, or native, to the functional entity.

The present invention additionally provides a recombinant targeting protein comprising (a) a surface loop from a first protein having a surface loop that specifically binds the target and (b) a functionalmotif or domain of a second protein.

The present invention provides a recombinant targeting protein wherein the surface loop is the HCDR3 of monoclonal antibody Fab-9, the second protein is human tissue type plasminogen activator (t-PA), and the target is platelet glycoprotein GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$). The present invention thus provides a platelet-targeting tissue plasminogen activator.

The present invention provides a method of reducing a blood clot in a subject comprising administering to the subject a therapeutic amount of a protein comprising (a) a surface loop from the HCDR3 of monoclonal antibody Fab-9 and (b) a functionalmotif or domain of human tissue type plasminogen activator (t-PA), thereby binding the protein to platelet glycoprotein GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$) on a platelet in a blood clot in the subject and reducing the blood clot in the subject.

The present invention further provides a method of preventing thrombosis or promoting thrombolysis in a subject comprising administering to the subject a therapeutic amount of a protein comprising (a) a surface loop from the HCDR3 of monoclonal antibody Fab-9 and (b) a functionalmotif or domain of human tissue type plasminogen activator (t-PA), thereby binding the protein to platelet glycoprotein GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$) on a platelet in a blood clot in the subject and preventing thrombolysis in the subject.

The present invention further provides a method of treating or preventing myocardial infarction in a subject comprising administering to the subject a therapeutic amount of a protein comprising (a) a surface loop from the HCDR3 of monoclonal antibody Fab-9 and (b) a functionalmotif or domain of human tissue type plasminogen activator (t-PA), thereby binding the protein to a platelet in the subject and treating or preventing myocardial infarction in the subject.

The present invention additionally provides a method of targeting a therapeutic compound to a tumor in a subject comprising administering to the subject a therapeutic agent comprising a targeted therapeutic or diagnostic functional entity linked to heavy chain complementarity determining region 3 (HCDR3) of monoclonal antibody Fab-9, wherein the therapeutic or diagnostic entity is an anti-tumor therapeutic compound

The present invention also provides a method of targeting a therapeutic protein to a tumor in a subject comprising administering to the subject a recombinant targeting protein comprising an anti-tumor therapeutic protein linked to the HCDR3 of monoclonal antibody Fab-9.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a representation of the NMR structure of murine epidermal growth factor adapted from Kohda et. al (28). Residue 23 of the murine protein corresponds to residue 65 of human t-PA. The β-turn formed by residues 23-28 of the murine protein may be extended in t-PA due to the occurrence of a three amino acid insertion, ^{67}YFS^{69}, at the location indicated by the large arrow. The primary sequence of the substituted region of LG-t-PA and the corresponding region of wild type t-PA are indicated at the bottom of the figure. Dashes in the LG-t-PA sequence indicate identity to the wild type enzyme.

Figure 2 shows standard indirect chromogenic assay of plasminogen activation by wild type and LG-t-PA in the presence of buffer (□), fibrin monomers (◇), fibrinogen (△), or cyanogen bromide fragments of fibrinogen (○).

Figure 3 shows the binding of LG-t-PA to platelet integrin $\alpha_{IIb}\beta_3$ which was measured using purified integrin as described in the Methods. Non-specific binding was determined by parallel incubation with 10 mM EDTA. The absolute amount of LG-t-PA bound was calculated based upon the specific activity of LG-t-PA.

Figure 4 demonstrates that RGD-containing peptide blocks the binding of LG-t-PA to platelet integrin $\alpha_{IIb}\beta_3$. A binding assay was performed as described in the examples. The binding of LG-t-PA to integrin $\alpha_{IIb}\beta_3$ was challenged with synthetic peptides of sequence GRGDSP (□) or SPGDRG (◇).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a targeted therapeutic or diagnostic agent comprising (a) a therapeutic or diagnostic functional entity linked to (b) an isolated targeting motif or domain that specifically binds a selected target and that is derived from or based on a protein or peptide binding region. Such targeting motifs or domains can include peptide mimetics or surface loops of proteins. Such targeting motifs or domains can include polyamino acids derived from binding regions of protein which binding regions have been isolated from the native protein and optimized to a higher affinity than the original binding region in its native protein. "Targeting motif or domain" is not intended to limit the motif or domain to any particular length or size or that it imply any particular secondary structure or any secondary structure at all, unless indicated.

For example, the present invention provides recombinant proteins wherein a surface loop has been grafted into a functional protein such that the protein retains its function(s) and has attained targeting capability of the surface loop that the surface loop imparted to its native protein. A specific example of this recombinant protein is an altered tissue type plasminogen activator, LG-t-PA, wherein the epidermal growth factor (EGF) domain of tissue type plasminogen activator was replaced with residues forming one CDR of a monoclonal antibody that was directed against the adhesive integrin receptor $\alpha_{IIb}\beta_3$ and had been subjected to mutagenesis and "affinity maturation" or "optimization" using a phage display system. The present invention further provides compositions comprising such recombinant proteins and nucleic acids encoding these recombinant proteins. Furthermore, the present invention provides methods of making and using these recombinant proteins, including methods of treating thrombolytic disorders and treating or preventing acute myocardial infarction.

A. General Considerations

An inherent limitation of many therapeutic and diagnostic agents is an inability to deliver these entities to specific sites, tissues, or pathological lesions within the body. Consequently, advances that facilitate precise and selective delivery of these entities, such as the present invention, promise to enhance the efficacy of many therapeutic and diagnostic regimens. Specific targeting is achieved in the current invention by obtaining and exploiting detailed information regarding protein-protein interactions.

Protein-protein interactions can be guided by contacts between surface loops within proteins. The present invention demonstrates that novel protein-protein interactions can be created using a strategy of "loop grafting" in which the amino acid sequence of a biologically active, flexible loop on one protein is used to replace a surface loop present on an unrelated protein.

To demonstrate this strategy, a surface loop within an epidermal growth factor module was replaced with the complementarity determining region (CDR) of a monoclonal antibody. Specifically, the HCDR3 from Fab-9, an antibody selected to bind the β3-integrins with nanomolar affinity (Smith, J.W., Hu, D., Satterthwait, A., Pinz-Sweeney, S., and Barbas, C.F. (1994); *J. Biol. Chem.* **269**, 32788-32795), was grafted into the EGF-like module of human tissue type plasminogen activator (t-PA). The resulting variant of t-PA bound to the platelet integrin $\alpha_{IIb}\beta_3$ with nanomolar affinity, retained full enzymatic activity, and was stimulated normally by the physiological co-factor fibrin. Binding of the novel variant of t-PA to integrin $\alpha_{IIb}\beta_3$ was dependent on the presence of divalent cations and was inhibited by an RGD-containing peptide, demonstrating that, like the donor antibody, the novel t-PA binds specifically to the ligand binding site of the integrin.

These findings demonstrate that surface loops within protein modules can be interchangeable and that phage display can be combined with loop grafting to direct proteins, at high affinity, to selected targets. These targets can include not only other proteins but also peptides, nucleic acids, carbohydrates, lipids, or even uncharacterized markers of specific cell types, tissues, or viruses.

The present examples demonstrate the feasibility of a protein loop grafting strategy. The resulting variant of t-PA, LG-t-PA, provides an improved thrombolytic agent for the treatment of acute myocardial infarction and other thromboembolic disorders. Previous efforts to target plasminogen activators to blood clots have often demonstrated that such targeting can enhance the thrombolytic potency of the plasminogen activator both in vitro and in vivo. LG-t-PA, therefore, due to its novel binding properties, can also be utilized for enhanced potency compositions to dissolve the platelet rich arterial thrombi which precipitate acute myocardial infarction.

B. Definitions

As used in the specification and in the claims, "a" can mean one or more, depending upon the context in which it is used.

An amino acid residue is an amino acid formed upon chemical digestion (hydrolysis) of a polypeptide at its peptide linkages. The amino acid residues described herein are preferably in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide. In keeping with the standard polypeptide nomenclature (described in J. Biol. Chem., 243:3552-59 (1969) and adopted at 37 CFR § 1.822(b) (2)), abbreviations for amino acid residues are shown in the following Table I:

TABLE I

SYMBOL		AMINO ACID
1 - Letter	3 - Letter	
Y	Tyr	tyrosine
G	Gly	glycine
F	Phe	phenylalanine
M	Met	methionine
A	Ala	alanine

	S	Ser	serine
	I	Ile	isoleucine
	L	Leu	leucine
	T	Thr	threonine
5	V	Val	valine

TABLE I (continued)

SYMBOL		AMINO ACID
1 - Letter	3 - Letter	
P	Pro	proline
K	Lys	lysine
H	His	histidine
Q	Gln	glutamine
E	Glu	glutamic acid
Z	Glx	Glu and/or Gln
W	Trp	tryptophan
R	Arg	arginine
D	Asp	aspartic acid
N	Asn	asparagine
B	Asx	Asn and/or Asp
C	Cys	cysteine
X	Xaa	Unknown or other

All amino acid residue sequences are represented herein by formulae whose left and right orientation is in the conventional direction of amino-terminus to carboxy-terminus. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino acid residues.

A "polypeptide" refers to a linear series of amino acid residues connected to one another by peptide bonds between the alpha-amino group and carboxy group of contiguous amino acid residues, and can be a protein.

A "peptide" refers to a linear series of less than or equal to about 50 amino acid residues, connected one to the other as in a polypeptide. A synthetic peptide is a chemically produced chain of amino acid residues linked together by peptide bonds.

A "polyamino acid" refers to a linear series of amino acid residues connected one to the other as in a polypeptide.

A protein refers to a linear series of greater than about 50 amino acid residues connected one to the other as in a polypeptide.

A "peptide mimetic" is defined to include a chemical compound, or an organic molecule, or any other peptide mimetic, the structure of which is based on or derived from a binding region of a protein. For example, one can model predicted chemical structures to mimic the structure of a binding region, such as a protein surface loop. Such modeling can be performed using standard methods. Alternatively, peptide mimetics can also be selected from combinatorial chemical libraries in much the same way that peptides are. (Ostresh, J.M. *et al., Proc Natl Acad Sci U S A* 1994 Nov 8;91(23):11138-42; Dorner, B. *et al., Bioorg Med Chem* 1996 May;4(5):709-15; Eichler, J. *et al., Med Res Rev* 1995 Nov;15(6):481-96; Blondelle, S.E. *et al. Biochem J* 1996 Jan 1;313 (Pt 1):141-7; Perez-Paya, E. *et al., J Biol Chem* 1996 Feb 23;271(8):4120-6)

A "surface loop" is defined as a binding, *i.e.*, targeting, element of a protein which element is a flexible loop structure in the native protein of about 2 to about 20 amino acids, that either connects regions of defined secondary structure in the native protein or connects a domain of secondary structure and a terminus of the native protein, and which element is selective for binding to one or more binding sites. A surface loop, as used in the claims, has retained one or more of its binding characteristics upon insertion into a non-native functional protein in a manner such that it remains as a surface loop, *e.g.*, such that it replaces a removed surface loop in the non-native functional protein or such that it is inserted either between two regions of defined secondary structure in the non-native functional protein or between a domain of secondary structure and a terminus in the non-native functional protein.

A "therapeutic or diagnostic functional entity" is defined as any medical, diagnostic, pharmaceutical or biological entity whose delivery to a targeted site in a subject has therapeutic benefit and/or diagnostic value and which entity can be linked to a peptide mimetic or polyamino acid. Examples of therapeutic or diagnostic functional entities include a nucleic acid; a protein; a peptide; a gene delivery vehicle (such as a plasmid, a virus, a liposome complex); a synthetic or naturally occurring enzyme (such as a protease, a phosphatase, a kinase, P450, or other drug metabolizing enzymes, superoxide dismutases or nitric oxide synthase); a thrombolytic agent (such as tissue plasminogen activator, uPA, vampire bat tPA, staphylokinase, streptokinase, an acylated streptokinase-plasminogen complex, or variants of any of the preceding); an anticoagulant (such as an inhibitor of the members of the blood coagulation cascade, antagonists of integrin adhesion receptors, protein C or activated protein C, tissue factor pathway inhibitor, or variants of blood coagulation enzymes like Factor VII, factor X or thrombin, inhibitors of platelet function, inhibitors of Factor XIII, compounds which modulate the activity of a protein involved in blood coagulation (for example, heparin), or any variant of the above); a chemotherapeutic agent (such as doxyrubicin (or an equivalent)); an apoptotic agent; a pharmaceutical; a chemical compound; a growth factor; a cytokine; other ligands for cell surface receptors; a carbohydrate, a lipid, uncharacterized markers and targets, drug delivery systems (e.g., miniaturized osmotic drug delivery pump), imaging agents, such as radiochemicals, fluorescence chemicals, metal ions that can be detected externally.

A target for binding of the therapeutic or diagnostic agent can be any region; tissue; organ; cell; virus; organelle; microorganism; a synthetic or naturally occurring molecule or macromolecule (such as a peptide, a protein, a lipid, a carbohydrate, a nucleic acid, as well as a modified variant thereof, such as a glycoprotein, a phosphoprotein, a glycolipid, a hormone); a protein (such as an enzyme and its inhibitors, a transcription factor, a kinase, a phosphatase, any protein found in the blood, an adhesive protein, a component of the extracellular matrix, a receptor or other cell surface protein, albumins, IgG-like molecules and antibodies, a growth factor, a cytokine and modulators of angiogenesis); a cell surface protein (such as an integrins, a

cadherin, a growth factor receptor, a proteoglycan, an ion channel, a member of the seven-membrane spanner protein family (such as odorant and taste receptors, the acetylcholine receptor)); a biological entity (such as a cell, a virus, a blood clot, a tumor, an component of a pathologic lesion (such as an aneurism, an atherosclerotic plaque), or a naturally occurring molecule or macromolecule). For example, a target can be integrin $\alpha_{IIb}\beta_3$, integrin $\alpha_v\beta_3$ or any other cell surface receptor.

A functional domain of a protein, which domain can be the therapeutic or diagnostic functional entity in an agent of this invention, is a region of, or an entire protein that has an activity desired to be targeted to a target in the subject. In some agents one activity has been isolated from its native protein or altered from its activity in its native protein. Additionally, functional domains derived from or isolated from more than one source can be linked together in an agent of this invention with a single or multiple targeting element.

By "optimized" as used in the claims is meant that a polyamino acid has been manipulated to increase its natural affinity for its target binding site. The term "optimized" is used to convey that the affinity and possibly the selectivity of the polyamino acid has been increased using a variety of standard approaches in the art. Optimization can also be referred to as "affinity maturation."

By "linked" is meant joined in a manner such that the function of the functional domain remains active and such that the targeting capability of the targeting motif or domain is available to target the agent. For example, when a surface loop is the targeting motif or domain and the functional entity is another protein, the surface loop can be inserted into the protein in a manner consistent with surface loops, *i.e.*, either between two regions of defined secondary structure in the non-native functional protein or between a domain of secondary structure and a terminus in the non-native functional protein. Linkages can also include any type of chemical bond, or peptide bonds. Linkages can include covalent and/or noncovalent bonds.

A composition comprising any agent, recombinant protein or nucleic acid can include a pharmaceutically acceptable carrier, for example, or any other selected addition that does not interfere with the desired function of the agent, protein or nucleic acid.

By administering an agent, protein, nucleic acid or composition is meant administering the agent, protein, nucleic acid or composition in a manner suitable for delivery to the selected target, as will be apparent to the artisan.

C. Therapeutic or diagnostic agents and compositions thereof

The present invention provides a targeted therapeutic or diagnostic agent comprising (a) a therapeutic or diagnostic functional entity linked to (b) an isolated targeting motif or domain that specifically binds a selected target and that is derived from or based on a protein or peptide binding region. At least the following three categories of targeting motifs or domains, derived from an entity other than the functional entity to which they are to be linked to form the therapeutic or diagnostic agent, can be utilized in this invention: (1) a peptide mimetic, i.e., a substance modeled on a peptide binding, (2) an isolated, optimized, high affinity polyamino acid, derived from a binding site from any selected protein, and (3) a surface loop isolated from a protein.

Specifically, the present invention provides a targeted therapeutic or diagnostic agent comprising (a) a therapeutic or diagnostic functional entity linked to (b) an isolated peptide mimetic that specifically binds a selected target. The invention additionally provides a targeted therapeutic or diagnostic agent comprising (a) a therapeutic or diagnostic functional entity linked to (b) an isolated, optimized, high-affinity polyamino acid that specifically binds a selected target. The invention further provides a targeted therapeutic or diagnostic agent comprising (a) a therapeutic or diagnostic functional entity linked to (b) an isolated protein surface loop that specifically binds a selected target, wherein the protein surface loop is not native/endogenous to the functional entity.

The therapeutic or functional entity is selected based upon the desired effect, whether therapeutic or diagnostic, and the targeting motif or domain is selected based upon the site to be targeted. By "specifically binds" is meant that the targeting motif or domain predominantly binds the target site, with minimal non-specific binding to other targets, *i.e.*, with minimal background. Specific binding further means that the targeting motif or domain can be used to selectively remove the target from a sample comprising the target. Specific binding by an antibody CDR means that the antibody can be used to selectively remove the factor from serum or inhibit the factor's biological activity and can readily be determined by radio immune assay (RIA), bioassay, or enzyme-linked immunosorbant (ELISA) technology. Many targeting elements are known and can be utilized in the present invention. When the targeting element is a peptide mimetic, the mimetic can be designed to specifically bind a specific site by modeling the mimetic on the conformation of the original binding peptide. These mimetics can be further optimized for higher affinity binding if desired, by any selected means.

When the targeting element is an isolated, optimized, high affinity polyamino acid, the element, several standard approaches are useful in optimizing the binding affinity and/or binding selectivity of a polyamino acid. For example, vast libraries comprising many variants of the polyamino acid can be displayed by several techniques that are well known in the art. These include, but are not limited to: (1) phage display (for reviews see Bradbury, A., Cattaneo, A (1995) The use of phage display in neurobiology. *Trends in Neuroscience* 18:243-249; Barbas, C.F. (1993) Recent advances in phage display. *Current Opinion in Biotechnology* 4:526-530); (2) polysome display (Mattheakis,L.C., Bhatt,R.R.,Dower,W.J. (1994) An in vitro polysome display system for identifying ligands from very large peptide libraries. *Proc. Natl. Acad. Sci. U.S.A.* 91, 9022-9026); (3) displaying peptides on plasmids (Cull, M.G. Miller, J.F., Schatz, P.J. (1992) Screening for receptor ligands using large libraries of peptides linked to the C-terminus of the lac repressor. *Proc. Natl. Acad. Sci. U.S.A.* 89:1865-1869); (4) synthetic peptide or peptidometic libraries (Pinilla C; Appel JR; Houghten RA. Investigation of antigen-antibody interactions using a soluble, non-support-bound synthetic decapeptide library composed of four trillion (4 x 10(12) sequences. *Biochem J* 1994 Aug 1;301 (Pt

3):847-53; Dooley CT; Chung NN; Wilkes BC; Schiller PW; Bidlack JM; Pasternak GW; Houghten RA An all D-amino acid opioid peptide with central analgesic activity from a combinatorial library. *Science* 1994 Dec 23;266(5193):2019-22). The optimal polyamino acids can then be obtained from this library by standard biochemical or biological selection. Alternatively, (5) the isolated polyamino acid could be optimized by chemical modification. For example, its structure could be modified using standard techniques such as combinatorial chemistry and medicinal chemistry.

When the targeting element is a surface loop, the surface loop is also specific for its target. Surface loops can be selected for desirable targeting characteristics. Many surface loop regions of proteins are already known from databases which contain the three dimensional structures of proteins, and such surface loops can readily be used in the present invention. The position of protein surface loops within proteins can also be predicted using structural algorithms. For example, a complementarity determining region of an antibody, monoclonal or polyclonal, directed against a target molecule of interest can be used as a surface loop. Furthermore, any protein having a targeting characteristic of interest can be structurally analyzed by standard means to determine various regions containing a surface loop and the surface loop thus identified. Such structural analysis includes NMR, crystallography or predictive algorithm. Additionally, if desired, the affinity of a surface loop for its target can be increased, by standard means.

Additional targeting motifs or domains, particularly for an agent to target a nucleic acid target, are binding domains derived from transcription factors, such as a steroid receptor; basal transcription factors (e.g. TFIID, etc.), and sequence specific DNA binding transcription factors (e.g., AP1, AP2, SP1, NF1, etc). Additional transcription factors are listed in, for example, computer databases such as that maintained by the National Center for Biotechnology Information (NCBI, Bethesda, MD) accessible through the BLAST program (see item 19 (TFD) for transcription factors; item 20 for eukaryotic promoter sequences).

The target can include a tissue, a organ, a cell, protein, a peptide, a nucleic acid, a carbohydrate, a lipid, any component of a pathological lesion, or uncharacterized markers and targets. For example, one can target cells of a tumor, metastatic cells, cells of the vasculature (such as endothelial cells and smooth muscle cells), cells of the lungs, muscle (such as smooth muscle cells, cardiac muscle, etc.), cells of the kidneys, blood cells (such as T-cells), cells of the bone marrow, such as stem cells, cells of bone, neurons and related neurological cells such as glial cells, brain cells, liver cells, or precursors of any selected cell, etc. by selecting a targeting motif or domain specific for the tissue or cells, such as a targeting motif or domain that specifically binds a cell surface receptor expressed on the cells of the organ or tissue. For example the target receptor can be integrin like $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha IIb\beta 3$. The target receptor can be a growth factor receptor, a hormone receptor, a cytokine receptor, and the like. The target receptor can be a growth factor-dependent receptor (e.g., epidermal growth factor, nerve growth factor, etc.). The target receptor can also be a ligand-dependent receptor (such as a steroid receptor, thyroid hormone receptor, retinoic acid receptor, retinoid X receptor, TCCD (dioxin) receptor, fatty acid activatable receptors, and the like) or a stimulus-dependent receptor (such as peroxisome proliferator-activated receptor). Many of these receptors or factors can be found listed in the book [Parker, M.G. (1993) *Steroid Hormone Action* (Oxford University Press, New York, pp. 210)], in a recent review article [Tsai, M.J. & O'Malley, B.W. (1994) *Annu. Rev. Biochem.* **63**, 451-486], and in the GenBank database, which will contain additional receptors as well as the complete nucleotide sequences of the genes and cDNAs. The target can also be a soluble protein like an antibody, a growth factor, or a serum protein. Nucleic acid targets can include, for example, a nucleic acid encoding a protein of interest, which expression is desired to alter by binding of a functional entity to the regulatory sequences or to the RNA encoded by the nucleic acid. For example, one can increase or inhibit expression of a nucleic acid, or one can alter the time of expression of the nucleic acid.

The functional entity can be selected from a wide array of such entities, and is selected based upon the target and the selected effect of the functional entity to be

delivered to the target. For example, one might wish to target to the region of a blood clot an enzyme, like t-PA, that will act to dissolve blood clots. Similarly, proteins, and their inhibitors, that act within the hemostatic clotting cascade could be targeted to clots. Similarly, drugs and proteins which act to modify platelet function could be targeted to platelets within the clot, or to circulating platelets which may ultimately become constituents of the clot.

The functional entity can be a nucleic acid, such as for gene therapy. Such nucleic acids can encode, for example, a therapeutic protein or peptide, or it can encode an antisense RNA. The functional entity can also include a gene delivery vehicle carrying a gene of interest. For example, one might wish to target gene delivery vehicles to endothelial cells, tumor cells or osteoclasts, (all of which express the $\alpha_v\beta_3$ integrin). One such gene delivery vehicle might be a virus, which could be but is not limited to, an adenovirus, an adeno-associated virus, an attenuated HIV virus and retroviruses, or another gene delivery vehicle such as a liposome or a lipid-based delivery vehicle. The types of genes which might be delivered to these sites could include genes that encode tumor suppressors, molecules which act to promote or prevent cell growth and/or cell migration. These genes could also include growth factors, cytokines and other various ligands for cell surface receptors which would exist in the immediate vicinity of the target.

The functional entity can further be, for example, drugs which act as poisons or apoptotic agents to cells to specific cell types. For example, one can target chemotherapeutic agents such as doxyrubicin (or an equivalent) to tumors with the intent of focusing the activity of the chemotherapeutic agent at the site of the tumor. Furthermore, one can also target drugs that act as poisons, or apoptotic agents to endothelial cells such that these poisons would kill endothelial cells in tumors, thereby eliminating the blood and nutrient supply to the tumor and killing it. Additionally, for example, one can also target a cell in the immune system, for example a cytotoxic T cell or a natural killer cell, to tumors, such that the cytotoxic T cell would recognize, bind to and kill the tumor cell by lysing it.

In another example, bone marrow transplantation, one can also target stem cells (functional entity) to the bone marrow (target) such that the stem cells would "home" to the bone marrow and establish residence, thereby increasing the "take" of the transplantation. In another example, one can also target drugs which act as bone sparing agents to the bone in order to prevent osteoporosis. One such example could be the bis-phosphonates, which act as poisons to the osteoclast, the major bone-resorbing cell.

Another type of functional entity which can be targeted to a site by the present method is a medical or diagnostic device. Such a device can include a drug delivery system, such as a miniaturized osmotic drug delivery pump, an imaging agent, such as a radiochemical, which enables the imaging of tumors or disease lesions using standard radiology techniques, an agent which can be targeted to tumors and other disease lesions such that it is detected externally by nuclear magnetic resonance or by magnetic resonance imaging (such as a spin-labeled probe, for example a metal ion like manganese).

The present invention further provides a recombinant targeting protein comprising (a) a surface loop from a first protein having a surface loop that specifically binds the target molcule and (b) a functional domain of a second protein. The second protein, supplying the functional domain of a recombinant, targeting protein of this invention, can be any selected protein that has a functional characteristic desired to be imparted to the recombinant protein, as described further herein. Placement of the surface loop into the second protein can be achieved by any of several standard methods, such as recombinant subcloning techniques to isolate the surface loop from a nucleic acid encoding the first protein having the surface loop and to insert the isolated nucleic acid into a nucleic acid encoding a functional protein. The region of DNA encoding the native surface loop of the functional protein can be removed prior to or during insertion of the non-native surface loop also by standard subcloning techniques and the non-native surface loop inserted. The recombinant DNA can then be expressed to produce the recombinant protein. Alternatively, the removed native surface loop of the functional

protein can be mutated to create a nucleic acid fragment corresponding in sequence to a targeting surface loop of the first protein having the surface loop and this mutated nucleic acid reinserted into the functional protein. Such mutations can be generated by standard techniques such as site-directed mutagenesis.

The present invention specifically provides a recombinant targeting protein wherein the surface loop is a complementarity determining region (CDR) of a monoclonal antibody directed against the target. Any desired monoclonal antibody can be utilized for this protein. The antibody can be directed against a cell surface protein, for example. The cell surface protein can be an integrin, such as platelet glycoprotein GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$) . Further more, the CDR can be further optimized for higher affinity, if desired, such as by methods described herein. In a specific example herein the monoclonal antibody Fab-9 has a CDR altered to contain a surface loop, and further, the surface loop has been optimized by phage display (the surface loop in the HCDR3 of monoclonal antibody Fab-9.).

The present invention additionally provides a recombinant targeting protein wherein the functional domain is human tissue type plasminogen activator (t-PA). A useful targeting motif or domain for the activator is a motif or domain that binds a receptor on a cell in or near a blood clot in an individual. For example, the target can be platelet glycoprotein GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$) or $\alpha_v\beta_3$ on surrounding vascular endothelial and smooth muscle cells, and thus the surface loop selected for the targeting motif or domain is one that specifically binds the receptor(s). For example, the present invention provides a recombinant molecule-targeting protein wherein the surface loop is the HCDR3 of monoclonal antibody Fab-9, the second protein is human tissue type plasminogen activator (t-PA), and the molecule targeted is platelet glycoprotein GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$). Thus, the present invention provides a platelet-targeting tissue plasminogen activator. This targeting protein is a clot-specific thrombolytic agent and can be used in therapies where dissolution of a clot is desirable.

In isolating and, if desired, optimizing, a surface loop for use in a targeted entity of the present invention, a nucleic acid encoding the region of a selected loop can be removed from the nucleic acid its native protein and inserted into a generic scaffold, such as a phage, polysome, or plasmid. The surface loop can be assayed for binding affinity to a target. If desired, the surface loop can be optimized, such as by mutagenesis and affinity maturation using, *e.g.*, phage display (*e.g.*, Pharmacia Biotech, Inc.'s Recombinant Phage Antibody System), polysome display, displaying peptides on plasmids, and using synthetic peptide libraries. These optimized biologically active motifs are then grafted, by standard methods, into several different positions of the recipient functional domain, *e.g.*, functional domain of a protein, to create a novel variant with altered binding properties. In particular, a selected surface loop can be inserted into a protein carrying a functional domain between two regions of defined secondary structure or between a domain of secondary structure and a terminus of the protein. Thus, the methods described herein can be used to modify the surface loops of a preselected protein binding (*i.e.*, targeting) motif or domain to form a high-affinity targeting module. Thereafter the targeting module can be fused with a preselected protein's functional domain module to form the targeting protein.

The present invention provides a composition comprising any therapeutic or diagnostic agent, recombinant protein or nucleic acid of this invention. Such a composition can include a pharmaceutically acceptable carrier, for example, physiological saline, or any other selected addition that does not interfere with the desired function of the agent, protein or nucleic acid.

Nucleic acids and cells

The present invention provides an isolated nucleic acid that functionally encodes any recombinant protein of this invention. By "isolated" as used herein is meant substantially free of, or separated from, other nucleic acids. Depending upon the context, it can mean substantially free of, or separated from, other nucleic acids naturally occurring in an organism containing the nucleic acid. Nucleic acids can be made by standard recombinant methods (*see. e.g.*, Sambrook et al., *Molecular Cloning: A*

Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989; *DNA cloning: A Practical Approach*, Volumes I and II, Glover, D.M. ed., IRL Press Limited, Oxford, 1985) and/or other standard methods, such as chemical synthesis. Nucleic acids can be produced in cells and isolated therefrom, if desired to be isolated from a cell. Alternatively, a cell can be used to produce the recombinant protein encoded by the nucleic acid. The nucleic acid can be in a plasmid, a virus, a phage, a cosmid, a yeast artificial chromosome or any other vector of convenience.

To functionally encode a protein, *i.e.*, allow the nucleic acid to be expressed, the nucleic acid can include, for example, expression control sequences, such as an origin of replication, a promoter, an enhancer, and necessary information processing sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences.

Additionally provided by the invention are nucleic acids that specifically hybridize to the nucleic acids encoding the recombinant proteins under sufficient stringency conditions to selectively hybridize to the nucleic acid. Thus, nucleic acids for use, for example, as primers and probes to detect or amplify the nucleic acids encoding the recombinant proteins are contemplated herein. Typically, the stringency of hybridization to achieve selective hybridization is about 5°C to 20°C below the Tm (the melting temperature at which half of the molecules dissociate from its partner). Hybridization temperatures are typically higher for DNA-RNA and RNA-RNA hybridizations. The washing temperatures can similarly be used to achieve selective stringency, as is known in the art. (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989; Kunkel et al. *Methods Enzymol.* 1987:154:367, 1987).

The present invention additionally provides a cell containing any nucleic acid of this invention. The cell can be generated by standard nucleic acid transfer methods, such as transfection, electroporation, calcium phosphate-mediated transfer, direct injection and the like. The cell can contain a vector containing the nucleic acid. A cell containing a

nucleic acid encoding a chimeric protein typically can replicate the DNA and, further, typically can express the encoded protein. The cell can be a prokaryotic cell, particularly for the purpose of producing quantities of the nucleic acid, or a eukaryotic cell, particularly a mammalian cell. The cell is preferably a mammalian cell for the purpose of expressing the encoded protein so that the resultant produced protein has mammalian protein processing modifications.

Methods of use

The present invention provides a method of reducing a blood clot in a subject comprising administering to the subject a therapeutic amount of a protein comprising (a) a surface loop from the HCDR3 of monoclonal antibody Fab-9 and (b) a functional domain of human tissue type plasminogen activator (t-PA), thereby binding the protein to platelet glycoprotein GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$) on a platelet in a blood clot in the subject and reducing the blood clot in the subject. The administered protein can function to (1) block integrin $\alpha_{IIb}\beta_3$, (2) localize and concentrate the t-PA and its activity to the clot by binding to the platelet and (3) localize t-PA to the clot by binding to $\alpha_v\beta_3$ expressed on vascular endothelial and smooth muscle cells in the vicinity of the clot. For such a method, intravenous or intraarterial administration of the protein can be particularly beneficial. Additional therapeutic proteins can be made that have a surface loop that binds specifically to other entities within the cardiovascular system, such as other components of blood and other components of blood clots and other surface receptors on platelets, vascular endothelial cells.

Thus, the present invention further provides a method of preventing thrombosis or promoting thrombolysis in a subject comprising administering to the subject a therapeutic amount of a protein comprising (a) a surface loop from the HCDR3 of monoclonal antibody Fab-9 and (b) a functional domain of human tissue type plasminogen activator (t-PA), thereby binding the protein to platelet glycoprotein GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$) on a platelet in a blood clot in the subject and preventing

thrombosis or promoting thrombolysis in the subject. For such a method, intravenous or intraarterial administration of the protein can be particularly beneficial.

The present invention further provides a method of treating or preventing myocardial infarction in a subject comprising administering to the subject a therapeutic amount of a protein comprising (a) a surface loop from the HCDR3 of monoclonal antibody Fab-9 and (b) a functional domain of human tissue type plasminogen activator (t-PA), thereby binding the protein to a platelet in the subject and treating or preventing myocardial infarction in the subject. Additionally, the present invention provides a method of treating or preventing myocardial infarction in a subject comprising administering to the subject a therapeutic amount of a targeted therapeutic agent of this invention having as the therapeutic functional entity any anti-platelet agent or anti-coagulant, linked to any targeting agent selective, or at least partially selective, for constituents of blood clots (for example, proteins and peptide mimetics that bind the $\alpha_{IIB}\beta_3$ integrin or any agent which binds the platelet). Administration is preferably performed by methods typical for administering therapeutics for myocardial infarction, and particularly by methods typical for administering t-PA, such as intravenously and intraarterially.

By "treating" a disease or condition is meant reducing or preventing any of the clinical manifestations of the disease or condition. For example, for treating myocardial infarction, the manifestations are known in the art and include chest pains, elevated circulating heart isozymes, alteration of an electrocardiogram, from occlusion of a coronary artery, and others, as known to the skilled artisan. One can monitor the effect of a therapeutic agent of this invention on the disease or condition being treated by detecting and/or, when possible, measuring any change in a clinical manifestation of the disease or condition by the appropriate means for detecting such change, many of which are known in the art.

The present invention additionally provides a method of targeting a therapeutic compound to a tumor in a subject comprising administering to the subject a therapeutic

agent comprising a targeted therapeutic or diagnostic functional entity linked to heavy chain complementarity determining region 3 (HCDR3) of monoclonal antibody Fab-9, wherein the therapeutic or diagnostic entity is an anti-tumor therapeutic compound. Anti-tumor therapeutic compounds can include apoptotic agents or poisons, such as chemotherapeutic agents, *e.g.*, doxyrubicin; tumor suppressors, *e.g.*, p53; and modulators of nucleic acid metabolism. The therapeutic agent comprising a targeted therapeutic or diagnostic functional entity linked to heavy chain complementarity determining region 3 (HCDR3) of monoclonal antibody Fab-9 can bind $\alpha_v\beta_3$, which is present on several tumor cells, including melanoma, breast cancer, ovarioan cancer. Such a method can be used to reduce or halt tumor growth and/or establishment and thus treat cancer.

The present invention also provides a method of targeting a therapeutic protein to a tumor in a subject comprising administering to the subject a recombinant tumor-targeting protein comprising an anti-tumor therapeutic protein linked to the HCDR3 of monoclonal antibody Fab-9. Such a method can be used to reduce or halt tumor growth and/or establishment and thus treat cancer.

The present invention also provides a method of targeting a therapeutic compound to an osteoclast in a subject comprising administering to the subject a targeted therapeutic agent comprising a therapeutic or diagnostic functional entity linked to heavy chain complementarity determining region 3 (HCDR3) of monoclonal antibody Fab-9, wherein the therapeutic or diagnostic entity is an anti-osteoporosis therapeutic compound. Examples of anti-osteoporosis therapeutic compounds include bis-phosphonates.

The present invention further provides a method of targeting a therapeutic protein to an osteoclast in a subject comprising administering to the subject, a recombinant osteoclast-targeting protein comprising an anti-osteoporosis therapeutic protein linked to the HCDR3 of monoclonal antibody Fab-9.

The present invention additionally provides a method of targeting a therapeutic compound to an endothelial cell which is in the process of angiogenesis in a subject comprising administering to the subject an endothelial cell- targeted agent comprising a therapeutic compound linked to a targeting motif or domain, such as a peptide mimetic, an optimized high-affinity polyamino acid, or isolated protein surface loop, which targeting motif or domain is selective for the $\alpha_v\beta_3$ integrin and in which the therapeutic compound is an anti-angiogenic factor or a cellular poison. For example, the targeting motif or domain can be the HCDR3 of monoclonal antibody Fab-9 or any other CDR of a monoclonal antibody that specifically binds $\alpha_v\beta_3$ integrin.

The present invention further provides a method of targeting a therapeutic compound to a tumor or tumor cell expressing $\alpha_v\beta_3$ integrin in a subject comprising administering to the subject a tumor-targeted agent comprising a therapeutic compound linked to a targeting motif or domain, such as a peptide mimetic, an optimized high-affinity polyamino acid, or isolated protein surface loop, which targeting motif or domain is selective for the $\alpha_v\beta_3$ integrin and in which the therapeutic compound is a cell with anti-tumor activity and which cell bears (or displays on its surface) the targeting motif or domain. The targeting motif or domain could be, for example, a targeting motif or domain grafted into the T cell receptor or any cell surface protein that is a member of the IgG protein family, or any cell surface protein which contains an epidermal growth factor-like module, or any cell surface protein that contains a fibronectin type III module. For example, HCDR 3 of Fab-9 can be grafted into the T cell receptor. Using a T cell expressing this loop-grafted T cell receptor, one can target a T cell to bind to a tumor cell expressing $\alpha_v\beta_3$ integrin .

The present invention further provides a method of targeting a therapeutic compound to a vascular smooth muscle cell (SMC) which is contributing to vascular stenosis in a subject comprising administering to the subject a SMC-targeted agent comprising a therapeutic compound linked to a targeting motif or domain, such as a peptide mimetic, an optimized high-affinity polyamino acid, or isolated protein surface

loop, which targeting motif or domain is selective for the $\alpha_v\beta_3$ integrin and in which the therapeutic compound is a modulator of cell growth, or a cellular poison.

For any method of this invention, the subject can be any animal, preferably a mammal, such as a human, a veterinary animal, such as a cat, dog, horse, pig, goat, sheep, or cow, or a laboratory animal, such as a mouse, rat, rabbit, or guinea pig. The therapeutic agent is selected accordingly to optimize the treatment for the subject being treated.

Diagnostic or therapeutic agents of the present invention can be administered to a subject or an animal model by any of many standard means for administering therapeutics or diagnostics to that selected site or standard for administering that type of functional entity. For example, an agent can be administered orally, parenterally (e.g., intravenously), by intramuscular injection, by intraperitoneal injection, topically, transdermally, or the like. Agents can be administered, *e.g.*, as a complex with cationic liposomes, or encapsulated in anionic liposomes. Compositions can include various amounts of the selected agent in combination with a pharmaceutically acceptable carrier and, in addition, if desired, may include other medicinal agents, pharmaceutical agents, carriers, adjuvants, diluents, etc. Parental administration, if used, is generally characterized by injection. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Depending upon the mode of administration, the agent can be optimized to avoid degradation in the subject, such as by encapsulation, etc.

Dosages will depend upon the mode of administration, the disease or condition to be treated, and the individual subject's condition, but will be that dosage typical for and used in administration of the functional domain. For example, for administering a protein comprising (a) a surface loop from the HCDR3 of monoclonal antibody Fab-9 and (b) a functional domain of human tissue type plasminogen activator (t-PA) to reduce a blood clot or to prevent thrombosis or promote thrombolysis or to treat or prevent

myocardial infarction, the protein can be administered in a dosage typical for t-PA. Such dosages are well -known in the art. Because of the efficient targeting of the recombinant protein of the present invention, this dosage can likely be reduced. Furthermore, the dosage can be adjusted according to the typical dosage for the specific disease or condition to be treated. Therefore, to reduce a blood clot, a typical dosage will be similar to or less than that administered when t-PA is administered to reduce a blood clot. To prevent thrombosis or prevent thrombolysis, a typical dosage will be similar to or less than that administered when t-PA is administered to prevent thrombosis or promote thrombolysis. To treat or prevent myocardial infarction a typical dosage will be similar to or less than that administered when t-PA is administered to treat or prevent myocardial infarction. Additionally, the dosage of a plasmid or virus can be that dosage typical for and used in administration of other plasmid or viral vectors (see e.g., U.S. Pat. No.4,897,355). Furthermore, culture cells or biopsy of the target cell type can be used to optimize the dosage for the target cells *in vivo*. Often a single dose can be sufficient; however, the dose can be repeated if desirable. The dosage should not be so large as to cause adverse side effects. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the patient and can be determined by one of skill in the art. The dosage can also be adjusted by the individual physician in the event of any complication.

Statement Concerning Utility

The present invention provides a targeted therapeutic or diagnostic agent comprising (a) a therapeutic or diagnostic functional entity linked to (b) an isolated targeting motif or domain that specifically binds a selected target and that is derived from or based on a protein or peptide binding region. Such agents can had widespread utility for therapy and diagnosis. The present invention can be adapted to any known treatment that includes administering a substance that functions in the subject at a site that can be targeted by this method, to improve the effectiveness of the treatment.

The present compositions and examples of use demonstrate specifically that surface loops within protein modules can be interchangeable and that phage display can be combined with loop grafting to direct proteins, at high affinity, to selected targets. A major finding of this study was that amino acids forming a biologically active, flexible surface loop on one protein could be grafted into another, unrelated protein and still maintain their initial binding activity. The protein sequence of the grafted loop was originally optimized by mutagenesis and affinity maturation of the donor protein, monoclonal antibody Fab-9, using phage display.

This invention differs significantly both in approach, and in end result, from previous methods, for example, previously reported studies where RGD sequences have been inserted into non-adhesive proteins (33-35) to target cells expressing receptors that bind RGD. Although these efforts have successfully targeted recipient proteins to integrins, substantial quantitation of binding affinities has not been reported for these proteins nor has the integrin target been identified. In these studies, an RGD sequence was inserted into either lysozyme or calpastatin, and the apparent k_D of the resulting, mutated protein for integrin appeared, based on cell adhesion or cell spreading assays, to be approximately 400 nM or 50 nM, respectively. In the present invention, a targeting molecule against integrin that possesses 50-400 fold higher affinity, with a k_D approaching one nanomolar, is prepared. The present invention shows that grafting the amino acid sequence of HCDR3 of the optimized antibody into the extended loop of an EGF module can transfer nanomolar affinity for integrin to the EGF domain. The high affinity of LG-t-PA for integrin almost certainly depends on maintenance of important biophysical properties of the loop; both linear and cyclic synthetic peptides containing the amino acid sequence of the Fab-9 HCDR3 exhibit approximately one-hundred fold lower affinity for integrins than LG-t-PA or Fab-9.

The development of a general method to accomplish the *ab initio* design of a three dimensional structure and underlying amino acid sequence of a peptide motif, or mimetic thereof, that can be placed into a chosen recipient protein, or other agent, and will bind, at high affinity, to a selected target molecule will be of great benefit. The inventive

method allows one to endow proteins with new, useful binding properties without resort to very large scale mutagenesis protocols followed by laborious and time consuming screening of individual variants of the protein of interest.

The present invention is more particularly described in the following examples which are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

EXAMPLES

Site directed mutagenesis and construction of expression vectors encoding variants of t-PA.

Oligonucleotide directed site specific mutagenesis was performed by the method of Zoller and Smith (15) as modified by Kunkel (16). To simplify later manipulations, we used as a template for mutagenesis a cDNA encoding t-PA (Pennica, D et al (1983) Nature vol. 310 pgs. 214-221; Sambrook et al (1986) Mol. Biol. & Medicine. vol 3, pgs 459-481) that contained a silent mutation at nucleotide 407 which created a new Kpn I site (17). The 437 bp Hind III - Kpn I bp fragment of this cDNA was subcloned into bacteriophage M13mp18 and site directed mutagenesis was performed using the mutagenic primer 5'-
CGGGGGCACCTGCTCATTCGGAAGGGGAGACATTAGGAATGTGTGCCAGTG
CC - 3' (SEQ ID NO:1).

Following mutagenesis, ssDNA corresponding to the entire 437 bp Hind III - Kpn I fragment was fully sequenced to assure the presence of the desired mutation and the absence of any additional mutations. Replicative form (RF) DNA was prepared for appropriate phage, and the mutated 437 bp fragment was recovered after digestion of RF DNA with Hind III and Kpn I and electrophoresis of the digestion products on an agarose gel. The isolated Hind III - Kpn I fragment was used to reconstruct a full length

cDNA encoding LG-t-PA. The surface loop generated in HCDR 3 of Fab-9 was separately grafted into the T cell receptor.

Expression of enzymes by transient transfection of Cos cells.

cDNAs encoding t-PA and LG-t-PA were ligated into the transient expression vector pSVT7 (18) and then introduced into Cos 1 cells by electroporation using a Bio Rad Gene Pulser. 20 μg of cDNA, 100 μg of carrier DNA and approximately 10^7 Cos cells were placed into a 0.4 cm cuvette, and electroporation was performed at 320 V, 960 μFD, and $\Omega = \infty$. Following electroporation, cells were incubated overnight at 37°C in DMEM containing 10% fetal calf serum and 5 mM sodium butyrate. Cells were then washed with serum free medium and incubated in DMEM for 48 hours at 37° C. After the incubation with serum free media, conditioned media were collected and enzyme concentrations were determined by ELISA.

Purification and Quantitation of Enzymes.

Conditioned media were dialyzed against 20 mM sodium phosphate (pH = 7.0), 100 mM NaCl, 20 mM arginine, and 0.05% Tween 80 and loaded onto a lysine-Sepharose (Pharmacia) column. The column was washed with 20 volumes of loading buffer, and t-PA was eluted with buffer containing 20 mM sodium phosphate (pH = 7.0), 100 mM NaCl, 200 mM arginine, and 0.05% Tween 80. Enzyme concentrations were measured by ELISA.

Kinetic Analysis of Plasminogen Activation using Indirect Chromogenic Assays.

Indirect chromogenic assays of t-PA utilized the substrates lys-plasminogen (American Diagnostica) and Spectrozyme PL (American Diagnostica) and were performed as previously described (18-20). Assays were performed in the presence of the co-factor DESAFIB (American Diagnostica). DESAFIB, a preparation of soluble fibrin monomers, was produced by digesting highly purified human fibrinogen with the protease batroxobin. Batroxobin cleaved the Arg 16 - Gly 17 bond in the Aα-chain of fibrinogen and consequently released fibrinopeptide A. The resulting des-AA-fibrinogen

or fibrin I monomers are soluble in the presence of the peptide Gly - Pro - Arg - Pro. The concentration of lys-plasminogen was varied from 0.0125 - 0.2 μM.

Indirect Chromogenic Assays in the Presence of Various Stimulators.

Standard indirect chromogenic assays were performed as previously described (18-20). Briefly, 0.25 - 1 ng of enzyme, 0.2 μM lys-plasminogen and 0.62 mM Spectrozyme PL were present in a total volume of 100 μl. Assays were performed either in the presence of buffer, 25 μg/ml DESAFIB, 100 μg/ml cyanogen bromide fragments of fibrinogen (American Diagnostica) or 100 μg/ml fibrinogen. Assays were performed in microtiter plates, and the optical density at 405 nm was read every 30 seconds for one hour in a Molecular Devices Thermomax. Reactions were performed at 37°C.

Direct Chromogenic Assays of t-PA activity using activated substrates.

Direct assays of t-PA activity utilized the substrate Spec t-PA (American Diagnostica) and were performed as previously described (21) except that 0.02% Tween 80 was included in the assay.

Integrin Purification and Integrin Binding Measurements

Integrin $\alpha_{IIb}\beta_3$ was purified from human platelets on RGD-peptide affinity columns as previously described (22, 23). Integrin $\alpha_v\beta_3$ was purified using antibody affinity chromatography with LM-609 conjugated to Sepharose as described (24). Both integrins were greater than 90% pure as judged by Coomassie blue staining of acrylamide gels. Binding of the novel t-PA to each integrin was measured with an assay adapted from the ligand receptor binding assays previously reported (25, 26). Purified integrin was immobilized in Titertek 96-well plates in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. For coating, the integrin was diluted to a concentration of 5 μg/ml. Integrin was allowed to coat plastic plates for 18 hours at 4°C. Following coating, the non-specific protein binding sites on the plate were blocked by incubation with 50 mM Tris-HCl, pH 7.4, 100 mM NaCl containing 20 mg/ml of

BSA. For binding measurements, conditioned media containing wild type or modified t-PA was diluted to the appropriate enzyme concentrations in binding buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.5 mM $CaCl_2$ containing 1 mg/ml of BSA). The t-PA was applied to integrin immobilized in microtiter wells and allowed to bind for 2 hours at 37°C. After binding, free t-PA was removed from the plate by three rapid washes with binding buffer and bound t-PA was detected using an activity assay for t-PA as previously described (21). Non-specific binding of t-PA was determined by the addition of 5 mM EDTA to chelate divalent cations that are required for integrin ligand binding function (23). In some cases the peptide GRGDSP (synthesized by Coast Scientific) was used as a competitor. This indirect assay was used for measuring t-PA binding to integrin because of technical limitations encountered using purified t-PA. Following purification, t-PA is maintained in solution with 200 mM arginine. We found that this concentration of arginine interfered with all of the integrin binding assays and precluded attempts to use direct methods of measuring binding affinity. Thus, the indirect binding assay was chosen. This type of measurement is expected to give an underestimate of the binding of loop-grafted t-PA to integrins because COS cells secrete other integrin binding proteins that compete with LG-t-PA for binding to integrin.

The Use of an Antibody CDR as a Donor and an EGF Module as a Recipient in Loop Grafting

The objective of this study was to test the concept that biologically active surface loops, at least in some cases, can be grafted between proteins of different backbone structure. As our donor we chose a CDR from Fab-9, a human antibody that we recently engineered to bind the ligand binding pocket of the β3-integrins (27). The HCDR3 loop from this antibody contains the active sequence SFGRGDIRN and is bounded by two cysteine residues. While Fab-9 binds to β3-integrins with nanomolar affinity, synthetic peptides with the HCDR3 sequence of Fab-9 display at least one hundred fold-lower affinity for integrin (14). The criteria for a successful grafting of the loop, therefore, is the retention of high affinity for integrin. An EGF module was chosen as a recipient because the structure of several proteins containing this module are available and show that EGF contains a large, exposed loop that contains a β-turn structure. This loop is

bounded by two disulfide pairs (28). Using oligonucleotide directed site specific mutagenesis and conventional cloning techniques, we constructed a cDNA encoding a variant of t-PA in which residues forming the exposed loop on the surface of the EGF domain have been replaced by amino acids found in the active loop of Fab-9 (Fig. 1). The Arg of the RGD motif was placed at residue 66 of t-PA, which, based upon NMR structures of EGF modules (28), is near the apex of this disulfide bounded loop. Because the CDR sequence of Fab-9 is one residue shorter than the EGF loop, we maintained the carboxy-terminal residue within the EGF loop as Val, the native residue at this position in t-PA. The new variant of t-PA is referred to as loop-grafted t-PA, or LG-t-PA.

The Modified t-PA Maintains Full Enzymatic Activity and is Stimulated by the Physiological Co-factor Fibrin.

A kinetic analysis of the activity of wild type and LG-t-PA toward the small chromogenic substrate Spec t-PA is summarized in Table IIa. The close correspondence between values of Km, k_{cat}, and k_{cat}/Km for the two enzymes in this assay clearly demonstrates that LG-t-PA maintains full activity towards synthetic substrates.

Table IIa. Kinetic constants for cleavage of the chromogenic substance Spec t-PA.

Enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}s^{-1}$)
t-PA	44	0.3	1.5×10^5
LG-t-PA	45	0.3	1.5×10^5

Table IIb presents the results of a kinetic assay of plasminogen activation, in the presence of the co-factor fibrin, by t-PA and LG-t-PA. The kinetic constants of LG-t-PA for plasminogen activation are very similar to those of wild type t-PA; k_{cat}/Km values for the two enzymes vary by approximately 10% in this assay. LG-t-PA, therefore, maintained full enzymatic activity not only toward small synthetic substrates but also towards the natural protein substrate plasminogen.

Table IIb. Activation of plasminogen in the presence of fibrin.

Enzyme	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}s^{-1}$)
t-PA	0.10	0.02	5.0×10^6
LG-t-PA	0.09	0.02	4.5×10^6

The activity of wild type t-PA is stimulated by fibrin, fibrinogen, and cyanogen bromide fragments of fibrinogen, and we and others have reported that mutations mapping to at least five distinct regions of the enzyme can differentially affect stimulation of t-PA by these distinct co-factors (29, 30). To examine whether mutations present in LG-t-PA affected stimulation of the enzyme by any of these three co-factors, we performed standard indirect chromogenic assays of the two enzymes in the presence of each of the co-factors. The results of these assays are depicted in Figure 2 and indicate that the mutations present in LG-t-PA have not significantly compromised interaction of the enzyme with any of the co-factors.

The role, if any, of the EGF domain of t-PA in mediating stimulation of the enzyme by fibrin has generated controversy and conflicting reports (9, 12, 31). Although this study will not resolve the issue, our results do argue strongly that residues 63 - 71, which form part of an antiparallel β-sheet and a β-turn on surface of the EGF domain, do not play a role in stimulation of the enzyme by fibrin or fibrinogen.

LG-t-PA Binds to β3-Integrins with Nanomolar Affinity

Two integrins containing the β3 subunit have been described. These are platelet integrin $\alpha_{IIb}\beta3$ and integrin $\alpha_v\beta_3$. Both of these integrins bind the antibody Fab-9 with nanomolar affinity (14, 27). The ability of the modified t-PA, LG-t-PA, to bind the two β3-integrins was tested using purified integrin and culture supernatant from cells transfected with the cDNA encoding LG-t-PA. An indirect binding assay was used to measure binding affinity of the LG-t-PA for integrins because purified t-PA cannot be maintained at high concentrations in buffers that are compatible with integrin binding assays (unpublished observation). Consequently, t-PA binding to integrin was measured by determining the amount of t-PA present in conditioned media from transfected COS

cells and using this media as a source of t-PA. t-PA that bound to purified integrin was reported as t-PA activity and was measured using a standard indirect chromogenic assay. The binding isotherm shown in Fig. 3 shows that the LG-t-PA binds purified integrin $\alpha_{IIb}\beta_3$. Non-specific binding was assessed by including EDTA in the binding study because ligand binding to integrins is dependent upon divalent cations (23, 32). The binding of LG-t-PA to $\alpha_{IIb}\beta_3$ is specific and saturable, exhibiting a K_D of approximately 0.9 nM. This k_D is the average of four similar experiments in which the dissociation constant ranged from 0.5 to 1.3 nM. In the experiment shown in Figure 3, the apparent k_D is 0.5 nM. The average k_D for this interaction compares favorably with the k_D of Fab-9 for this integrin of 5 nM (14). Like the protein containing the parent loop, Fab-9 , the modified t-PA also bound to integrin $\alpha_v\beta_3$. The affinity of the modified t-PA for this integrin was also high, with an apparent k_D of 1.8 nM, similar to the k_D of 1.7 nM exhibited by Fab-9. Thus, by contrast to linear and cyclic synthetic peptides with sequences that are identical to the HCDR3 of Fab-9, LG-t-PA maintained the high affinity for integrins exhibited by Fab-9.

Loop-grafted t-PA Binds to the Ligand Binding Site of β3-Integrins

One of the hallmarks of ligand binding to the β3-integrins is that small synthetic peptides with the RGD sequence can block their binding. The ability of RGD peptides to block LG-t-PA was tested by a simple competition assay. A concentration range of RGD peptide was included during the binding reaction with the modified t-PA. As a control, a synthetic peptide with the same composition, but random sequence was also used as competitor. Bound t-PA was detected with the indirect activity assay described in Methods. As shown in Fig. 4, the peptide with sequence GRGDSP blocked the binding of LG-t-PA to integrin, but a peptide with random sequence had no effect on binding. The data shown are for integrin $\alpha_{IIb}\beta_3$, and nearly identical data were obtained when RGD peptide was used to block binding of LG-t-PA to purified integrin $\alpha_v\beta_3$. These data show that, like Fab-9, LG-t-PA binds to the ligand binding site of the β3-integrins.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

Although the present process has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention except as and to the extent that they are included in the accompanying claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: TARGETED THERAPEUTIC OR DIAGNOSTIC AGENTS AND METHODS OF MAKING AND USING SAME

(iii) NUMBER OF SEQUENCES: 1

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(v) COMPUTER READABLE FORM:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGGGGGCACC TGCTCATTCG GAAGGGGAGA CATTAGGAAT GTGTGCCAGT GCC 53

What is claimed is:

1. A targeted therapeutic or diagnostic agent comprising (a) a therapeutic or diagnostic functional entity linked to (b) an isolated peptide mimetic that specifically binds a selected target.

2. A targeted therapeutic or diagnostic agent comprising (a) a therapeutic or diagnostic functional entity linked to (b) an isolated, optimized, high-affinity polyamino acid that specifically binds a selected target.

3. A targeted therapeutic or diagnostic agent comprising (a) a therapeutic or diagnostic functional entity linked to (b) an isolated naturally occuring or optimized protein surface loop that specifically binds a selected target, wherein the protein surface loop is not endogenous to the functional entity.

4. The targeted agent of any of claims 1, 2 or 3, wherein the functional entity is a medical or diagnostic device.

5. The targeted agent of any of claims 1, 2 or 3, wherein the entity is a cell, virus, gene delivery vehicle or a biological molecule.

6. The targeted agent of any of claims 1, 2 or 3, wherein the entity is a synthetic or naturally occurring macromolecule.

7. The targeted agent of any of claims 1, 2 or 3, wherein the entity is a synthetic or naturally occurring peptide or protein.

8. The targeted agent of any of claims 1, 2 or 3, wherein the entity is a synthetic or naturally occurring enzyme.

9. The targeted agent of any of claims 1, 2 or 3, wherein the entity is a thrombolytic agent or an anticoagulant.

10. The targeted agent of any of claims 1, 2 or 3, wherein the entity is a plasminogen activator.

11. The targeted agent of any of claims 1, 2 or 3, wherein the entity is tissue type plasminogen activator (tPA), or a variant of tissue type plasminogen activator.

12. The targeted agent of any of claims 1, 2 or 3, wherein the entity is loop-grafted tissue type plasminogen activator (LG-tPA).

13. The targeted agent of any of claims 1, 2 or 3, wherein the target is a biological entity.

14. The targeted agent of any of claims 1, 2 or 3, wherein the target is an organ, tumor, tissue, cell, virus, or microorganism.

15. The targeted agent of any of claims 1, 2 or 3, wherein the target is a synthetic or naturally occurring macromolecule.

16. The targeted agent of any of claims 1, 2 or 3, wherein the target is a protein.

17. The targeted agent of any of claims 1, 2 or 3, wherein the target is a cell surface protein.

18. The targeted agent of any of claims 1, 2 or 3, wherein the target is an integrin.

19. The targeted agent of any of claims 1, 2 or 3, wherein the target is an integrin that binds to an Arg-Gly-Asp (RGD) tripeptide motif.

20. The targeted agent of any of claims 1, 2 or 3, wherein the target is α_{I} I $b\beta_{I}$ integrin.

21. The targeted agent of any of claims 1, 2 or 3, wherein the target is $\alpha_{V}\beta_{I}$ integrin.

22. The targeted agent of claim 2, wherein the optimized, high affinity polyamino acid is a complementarity determining region of an IgG-like molecule.

23. The targeted agent of claim 2, wherein the optimized, high affinity polyamino acid is a complementarity determining region of an antibody molecule.

24. The targeted agent of claim 23, wherein the complementarity determining region is heavy chain complementarity determining region 3 (HCDR3) of monoclonal antibody Fab-9.

25. A recombinant targeting protein comprising (a) a surface loop from a first protein having a surface loop that specifically binds the target and (b) a functional domain of a second protein.

26. The recombinant targeting protein of claim 25, wherein the surface loop is a complementarity determining region of a monoclonal antibody directed against the target.

27. The recombinant targeting protein of claim 26, wherein the antibody is directed against a cell surface protein.

28. The recombinant targeting protein of claim 27, wherein the cell surface protein is an integrin.

29. The recombinant targeting protein of claim 28, wherein the integrin is platelet glycoprotein GPIIb/IIIa (integrin α_I I $b\beta_I$).

30. The recombinant targeting protein of claim 28, wherein the integrin is $\alpha_V\beta_I$.

31. The recombinant targeting protein of claim 29 or 30, wherein the surface loop is the HCDR3 of monoclonal antibody Fab-9.

32. The recombinant targeting protein of claim 25, wherein the second protein is human tissue type plasminogen activator (t-PA).

33. The recombinant targeting protein of claim 25, wherein the surface loop is the HCDR3 of monoclonal antibody Fab-9, the second protein is human tissue type plasminogen activator (t-PA), and the target is platelet glycoprotein GPIIb/IIIa (integrin α_I I $b\beta_I$).

34. A composition comprising the recombinant targeting protein of claim 25.

35. The composition of claim 34, further comprising a pharmaceutically acceptable carrier.

36. A composition comprising the recombinant targeting protein of claim 33.

37. The composition of claim 36, further comprising a pharmaceutically acceptable carrier.

38. An isolated nucleic acid encoding the recombinant protein of claim 25.

39. An isolated nucleic acid encoding the recombinant protein of claim 33.

40. An isolated nucleic acid that specifically hybridizes to the nucleic acid of claim 38.

41. An isolated nucleic acid that specifically hybridizes to the nucleic acid of claim 39.

42. A composition comprising the nucleic acid of claim 38.

43. The composition of claim 42, further comprising a pharmaceutically acceptable carrier.

44. A composition comprising the nucleic acid of claim 39.

45. The composition of claim 44, further comprising a pharmaceutically acceptable carrier.

46. A method of reducing a blood clot in a subject comprising administering to the subject a therapeutic amount of the protein of claim 33, thereby binding the protein to platelet glycoprotein GPIIb/IIIa (integrin α_{I} I $b\beta_{I}$) on a platelet in a blood clot in the subject and reducing the blood clot in the subject.

47. A method of preventing thrombosis or promoting thrombolysis in a subject comprising administering to the subject a therapeutic amount of the protein of claim 33, thereby binding the protein to platelet glycoprotein GPIIb/IIIa (integrin α_{I} I $b\beta_{I}$) on a platelet in a blood clot in the subject and preventing thrombosis or promoting thrombolysis in the subject.

48. A method of treating or preventing myocardial infarction in a subject comprising administering to the subject a therapeutic amount of the protein

of claim 33, thereby binding the protein to a platelet in the subject and treating or preventing myocardial infarction in the subject.

49. A method of targeting a therapeutic compound to a tumor in a subject comprising administering to the subject the targeted agent of claim 24, wherein the therapeutic or diagnostic entity is an anti-tumor therapeutic compound.

50. A method of targeting a therapeutic protein to a tumor in a subject comprising administering to the subject the protein of claim 31, wherein the second protein is an anti-tumor therapeutic protein.

51. A method of targeting a therapeutic compound to an osteoclast in a subject comprising administering to the subject the targeted agent of claim 24, wherein the therapeutic or diagnostic entity is an anti-osteoporosis therapeutic compound.

52. A method of targeting a therapeutic protein to an osteoclast in a subject comprising administering to the subject the protein of claim 31, wherein the second protein is an anti-osteoporosis therapeutic protein.

53. A method of targeting a therapeutic compound to an endothelial cell which is in the process of angiogenesis in a subject comprising administering to the subject the targeted agent of claim 24, wherein the therapeutic or diagnostic entity is an anti-angiogenic factor or a cellular poison.

54. A method of targeting a therapeutic compound to a tumor or tumor cell expressing $\alpha_V\beta_I$ integrin in a subject comprising administering to the subject the targeted agent of claim 24, wherein the therapeutic or diagnostic entity is a cell with anti-tumor activity.

55. A method of targeting a therapeutic compound to a vascular smooth muscle cell (SMC) which is contributing to vascular stenosis in a subject comprising administering to the subject the targeted agent of claim 24, wherein the therapeutic or diagnostic entity is a modulator of cell growth or a cellular poison.

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(54) Title: TARGETED THERAPEUTIC OR DIAGNOSTIC AGENTS AND METHODS OF MAKING AND USING SAME

(57) Abstract

The present invention provides a targeted therapeutic or diagnostic agent comprising (a) a therapeutic or diagnostic functional entity linked to one of the following: (a) an isolated peptide mimetic that specifically binds a selected target; (b) an isolated, optimized, high-affinity polyamino acid that specifically binds a selected target; (c) an isolated protein surface loop that specifically binds a selected target, wherein the protein surface loop is not endogenous to the functional entity. The invention additionally provides methods of targeting therapeutic or diagnostic agents to a target. Additionally provided is a method of targeting a therapeutic agent to a platelet using the present agents, and methods of treating diseases and disorders associated with blood clots. Specifically provided is a recombinant targeting protein wherein the surface loop is the HCDR3 of monoclonal antibody Fab-9, the second protein is human tissue type plasminogen activator (t-PA), and the target is platelet glycoprotein GPIIb/IIIa (integrin αIIbβI).

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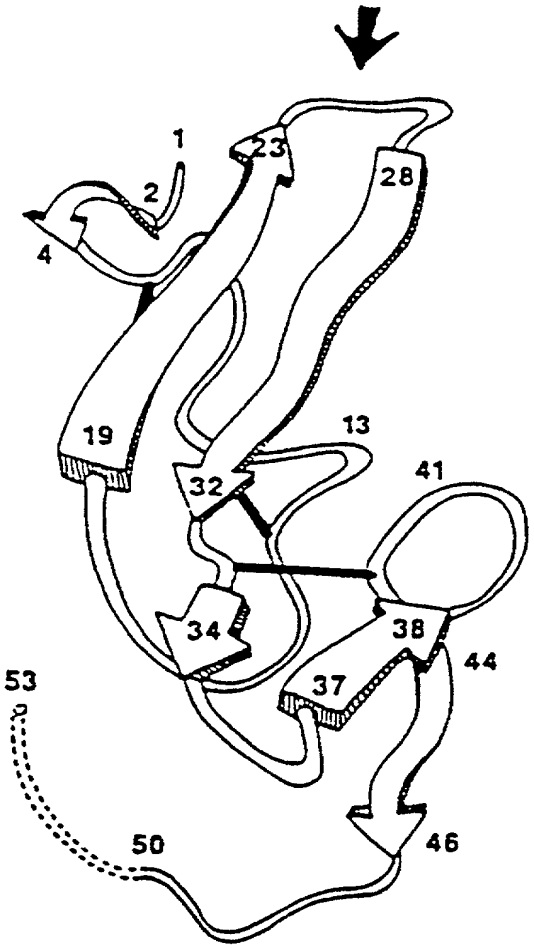

FIGURE 1

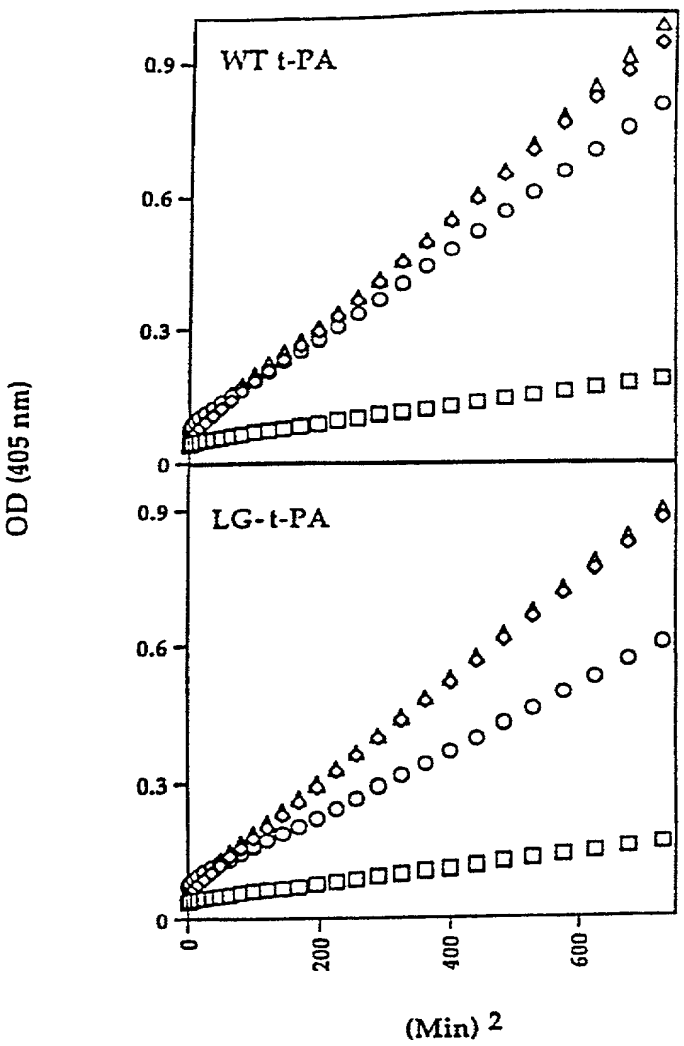

FIGURE 2

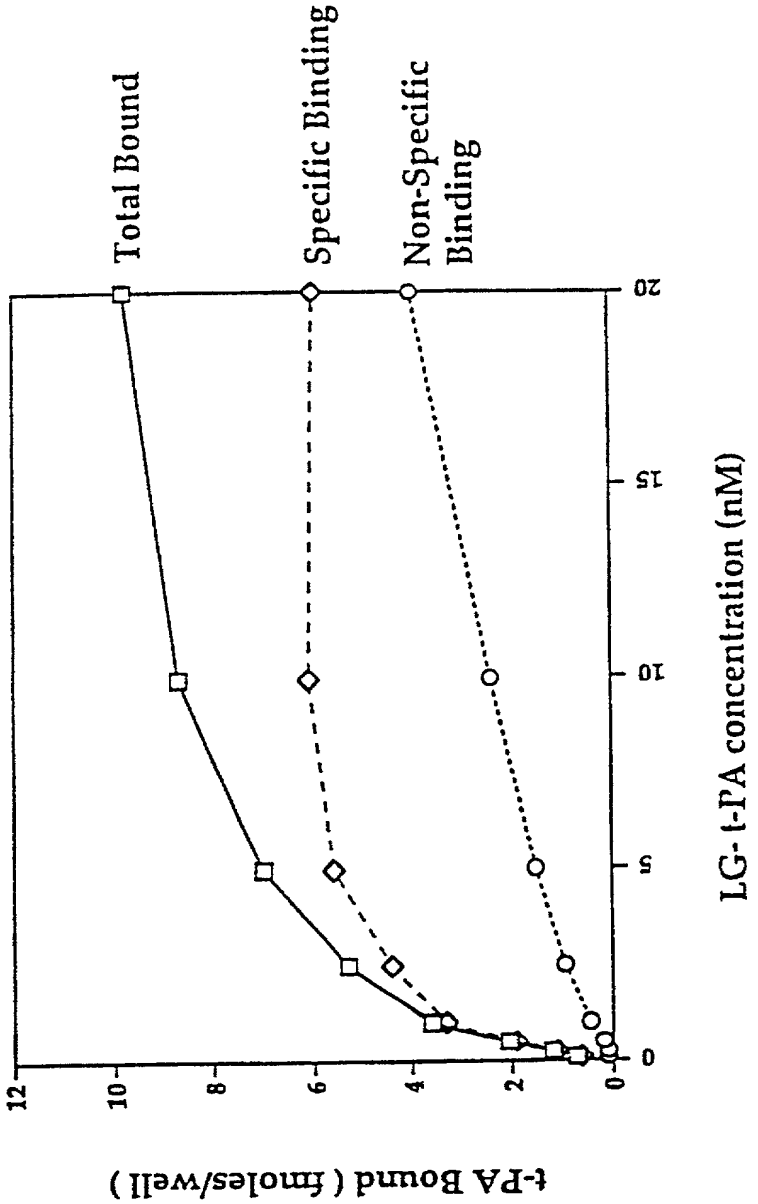
Total Bound
Specific Binding
Non-Specific Binding
t-PA Bound (fmoles/well)
0
2
4
6
8
10
12
0
5
10
15
20
LG- t-PA concentration (nM)

FIGURE 3

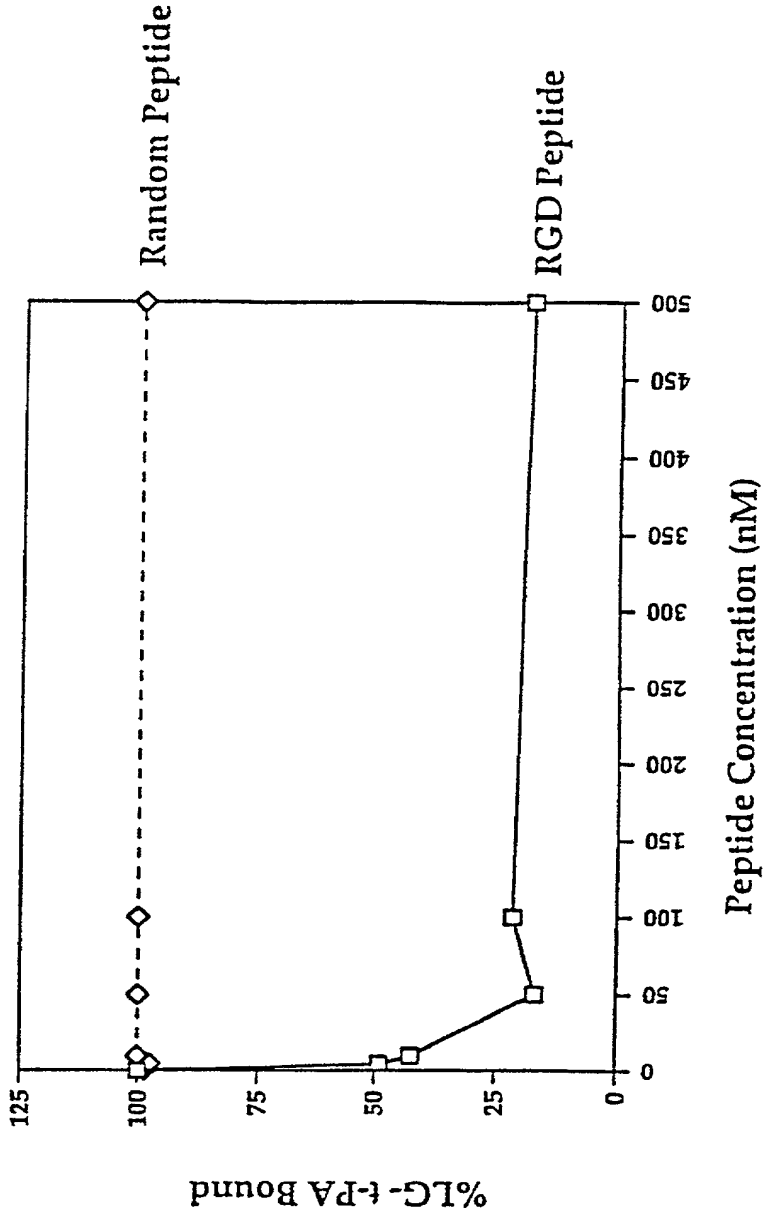
Random Peptide
RGD Peptide
Peptide Concentration (nM)
0
50
100
150
200
250
300
350
400
450
500
%LG-t-PA Bound
125
100
75
50
25
0

FIGURE 4

ATTORNEY DOCKET NO. 19191.0002

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

(X) Original () Supplemental () Substitute () PCT

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **"TARGETED THERAPEUTIC OR DIAGNOSTIC AGENTS AND METHODS OF MAKING AND USING SAME "**, which is described and claimed in the specification

(check one) [] which is attached hereto, or
[] which was filed on , as United States Application No. and with amendments through (if applicable), or
[X] in International Application No. PCT/US96/20577, filed December 19, 1996.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information known by me to be material to the patentability of the claims of this application in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a) - (d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate relating to this subject matter having a filing date before that of the application on which priority is claimed:

PRIOR FOREIGN APPLICATIONS: (ENTER BELOW IF APPLICABLE)			PRIORITY CLAIMED (MARK APPROPRIATE BOX BELOW)	
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PCT/US96/ 20577	PCT	19 DEC 1996	X	

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

APPLICATION NUMBER	FILING DATE
60/009,028	21 December 1995

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose all information known by me to be material to the patentability of the claims of this application as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NO.	FILING DATE	STATUS (MARK APPROPRIATE COLUMN BELOW)		
		PATENTED	PENDING	ABANDONED

I hereby appoint the following attorneys and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

William H. Needle (Reg. No. 26,209); Sumner C. Rosenberg (Reg. No. 28,753); David G. Perryman (Reg. No. 33,438); Mitchell A. Katz (Reg. No. 33,919); Gregory J. Kirsch (Reg. No. 35,572); D. Andrew Floam (Reg. No. 34,597); Gwendolyn D. Spratt (Reg. No. 36,016); Clark G. Sullivan (Reg. No. 36,942); Elizabeth Selby (Reg. No. 38,298); Yahn W. Bernier (Reg. No. 38,078); Mary L. Miller (Reg. No. 39,303); Bradley K. Groff (Reg. No. 39,695); Kean J. DeCarlo (Reg. No. 39,954); Allan G. Altera (Reg. No. 40,274); Bryan W. Bockhop (Reg. No. 39,613); Elket V. Swope (Reg. No. 40,195); J. William Seanor (Reg. No. 40,804), Robert T. Barker (Reg. No. P-41,597); Mark A. Westhafer (Reg. No. P-42,220); and Janice A. Kimpel (Reg. No. P-42,734).

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[] which was filed on , as United States Application No. and with amendments through (if applicable), or
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Inventor's signature: Edwin L Madison Date: 7/28/98

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Post Office Address: 615 Stratford Court #3, Del Mar, California 92014

Citizenship: USA

Full name of second inventor: SMITH, JEFFREY W.

Inventor's signature: _____ Date: _____

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Citizenship: USA

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Full name of first inventor: MADISON, EDWIN L.

Inventor's signature: _____ Date: _____

Residence: 615 Stratford Court #3, Del Mar, California 92014

Post Office Address: 615 Stratford Court #3, Del Mar, California 92014

Citizenship: USA

Full name of second inventor: SMITH, JEFFREY W.

Inventor's signature: Jeffrey W Smith Date: 7/27/98

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Citizenship: USA